CXXXVIII. A COLORIMETRIC METHOD FOR THE DETERMINATION OF *N*-ACETYLGLUCOSAMINE AND *N*-ACETYLCHONDROSAMINE.

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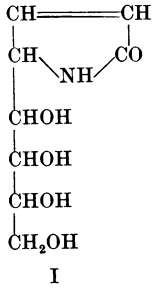
THE reaction of acetylated glucosamine with *p*-dimethylaminobenzaldehyde was first observed by Müller [1901] who showed that after warming a solution of penta-acetylglucosamine with dilute potassium hydroxide an intense reddish-purple colour was produced by the addition of a solution of *p*-dimethylaminobenzaldehyde acidified with hydrochloric acid [Ehrlich's reagent; 1901].

A colorimetric method for the quantitative estimation of *N*-acetylglucosamine, based on the above reaction, has recently been described by Zuckerkandl and Messiner-Klebermass [1931]. These workers suggested that when *N*-acetylglucosamine (II) is warmed with dilute alkali the aldehyde group and the acetyl group react together with the subsequent formation of a pyrrole derivative (I) which then condenses with *p*-dimethylaminobenzaldehyde to yield the characteristic red coloration which pyrroles are known to give with Ehrlich's reagent. We have obtained evidence [Elson and Morgan, 1933, 1, 2, and unpublished results], however, that *N*-acetylglucosamine reacts in the enolic form (III) and passes over with loss of water into the oxazole derivative, 2-methyl-4-$\alpha\beta\gamma\delta$-tetrahydroxy-*n*-butyloxazole (IV), and we consider that it is the condensation of this substance with *p*-dimethylaminobenzaldehyde in acid solution which gives rise to the intense reddish purple coloration. The action of dilute alkali on *N*-acetylglucosamine will be considered in more detail in a later communication.



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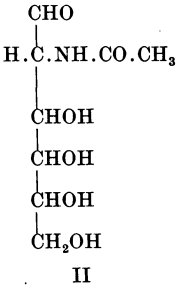

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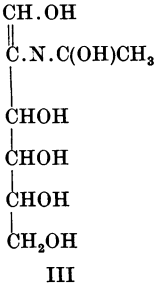

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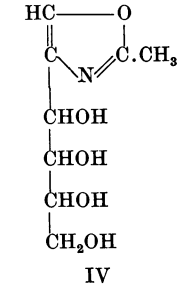

IV

According to the procedure of Zuckerkandl and Messiner-Klebermass *N*-acetylglucosamine (1–4 mg.), after dissolution in 5 ml. of 60 % aqueous alcohol, is heated for 6–8 seconds at boiling-point with two drops of 30 % potassium hydroxide solution and rapidly cooled. The *p*-dimethylaminobenzaldehyde reagent (1 ml.) is then added and after 10–15 minutes the reddish-

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purple colour which develops is matched colorimetrically against the colour produced by a standard solution of *N*-acetylglucosamine which has been treated in the same manner.

In our experience this method cannot be used where accurate and reproducible results are required. It is not proposed to discuss here the numerous variations in technique which have been tried in order to render the method suitable for our requirements, but only to state that investigations were carried out to determine the optimum conditions under which *N*-acetylglucosamine could be converted into the oxazole derivative (IV) and to ascertain the factors which are necessary for the production of the maximum colour intensity per mg. of the oxazole derivative when this compound is condensed with *p*-dimethylaminobenzaldehyde.

Early in our work it was found that unless the operation of boiling the acetylglucosamine in alkaline alcoholic solution were very carefully controlled a considerable proportion remained unconverted into the oxazole derivative or was destroyed by the action of the alkali. In view of the marked influence of potassium hydroxide upon the quantitative conversion of the *N*-acetylhexosamine into the corresponding oxazole under these conditions we consider that—"two drops of 30 % potassium hydroxide"—and—"heating for 6–8 seconds at boiling-point"—as recommended by Zuckerkandl and Messiner-Klebermass, do not define the concentration of alkali present in the test solution, or the time of heating, with sufficient accuracy for a quantitative procedure.

A greater colour intensity per mg. of acetylglucosamine results when the colour reaction is carried out in 90 % acetic acid solution and by the use of this solvent it has been possible to estimate quantities of acetylglucosamine as small as 0·08 mg. A perceptible colour is obtained with 0·01 mg. acetylglucosamine in a volume of 10 ml.; the test is therefore sensitive at a dilution of 1 in a million of acetylglucosamine.

Experimental.

Reagents.

(1) p-*Dimethylaminobenzaldehyde reagent.* *p*-Dimethylaminobenzaldehyde (A. R.) (2 g.) after two crystallisations from dilute acetic acid is dissolved in 100 ml. of glacial acetic acid containing 5·0 ml. of hydrochloric acid (A. R.). The final solution should possess a pale yellow colour. The addition of 1 ml. of water to 9 ml. of the reagent should not produce an increase in the intensity of the yellow colour. Some preparations of *p*-dimethylaminobenzaldehyde (A. R.) have been found to give yellow-coloured acetic-hydrochloric acid solutions which produce bright yellow solutions on the addition of water; these must be avoided. The reagent keeps indefinitely.

(2) N-*Acetylglucosamine standard.* *N*-Acetylglucosamine (1 g.) is dissolved in about 10 ml. of water saturated with chloroform and made up to a volume of 100 ml. From this standard solution dilutions which will contain a required amount of substance per ml. can readily be made. The standard solution should be kept at 0°.

(3) *0·5* N *sodium carbonate solution.*

Procedure.

A solution of *N*-acetylglucosamine, containing 0·1–1·0 mg. per ml., is measured into test-tubes of approximately equal thickness graduated at 10 ml.; if the volume of solution taken is less than 1·0 ml. it should be made up to 1·0 ml. by the addition of water. The sodium carbonate (0·1 ml.) is added and

the tubes are heated in a boiling water-bath for 5 minutes and then cooled. Standard solutions of *N*-acetylglucosamine are treated in the same manner. Glacial acetic acid is run into the tubes from a burette or separating funnel until it is within about 2 ml. of the 10 ml. mark. The *p*-dimethylaminobenzaldehyde reagent (1 ml.) is then added and the solution is made up to 10 ml. with acetic acid, thoroughly mixed and allowed to stand. The colour develops quickly and reaches its maximum intensity in about 45 minutes. When the maximum colour reached is compared with a stable artificial colour standard it shows no appreciable fading over a period of at least 1 hour.

As with most colorimetric methods, this method gives good results only when the colour intensities of the standard and the unknown solutions are approximately the same. Table I shows the results obtained by using the above

Table I.

N-Acetylglucosamine				*N*-Acetylglucosamine			
Standard mg.	Present mg.	Found mg.	% error	Standard mg.	Present mg.	Found mg.	% error
0·90	1·00	1·00	0·0	0·400	0·350	0·355	+1·4
,,	0·95	0·96	+1·0	,,	,,	0·350	0·0
,,	,,	0·97	+2·1	0·350	0·400	0·405	+1·2
,,	0·85	0·84	−1·2	,,	0·300	0·310	+3·3
,,	,,	0·86	+1·2	0·325	0·350	0·345	−1·4
,,	,,	,,	+1·2	,,	0·300	0·305	+1·7
,,	0·80	0·81	+1·3	0·300	0·275	0·275	0·0
0·80	0·90	0·89	−1·1	,,	0·250	0·255	+2·0
,,	0·70	0·70	0·0	0·225	,,	,,	+2·0
0·70	0·80	0·78	−2·5	0·175	0·200	0·205	+2·5
,,	,,	0·79	−1·2	0·150	0·175	0·174	−0·6
,,	0·65	0·64	−1·5	,,	,,	0·177	+0·6
,,	0·60	0·62	+3·3	0·090	0·100	0·102	+2·0
,,	,,	0·61	+1·6	,,	,,	0·099	−1·0
0·55	,,	0·60	0·0	,,	,,	0·101	+1·0
,,	,,	0·585	−2·5	,,	0·080	0·078	−2·5
,,	0·50	0·510	+2·0	,,	,,	,,	−2·5
,,	,,	0·500	0·0	,,	,,	0·079	−1·2
0·450	,,	0·510	+2·0				
,,	,,	0·495	−1·0				
,,	0·40	0·395	−1·2				

procedure when the solutions to be estimated differ in their *N*-acetylglucosamine content by not more than 20 % from that of the standard solution. The method is equally applicable to the estimation of *N*-acetylchondrosamine and it has been

Table II.

N-Acetylchondrosamine			
Standard mg.	Present mg.	Found mg.	% error
0·90	1·00	1·02	+2·0
,,	,,	1·00	0·0
,,	0·85	0·84	−1·2
,,	,,	0·86	+1·2
0·60	0·65	0·65	0·0
,,	,,	0·64	−1·5
,,	0·55	0·56	+1·8
0·550	0·500	0·505	+1·0
0·400	0·450	0·445	−1·1
,,	0·350	0·365	+4·3
,,	,,	0·360	+2·9
0·090	0·100	0·101	+1·0
,,	,,	0·102	+2·0

found that equal amounts of *N*-acetylglucosamine and *N*-acetylchondrosamine when compared under similar conditions give rise to colours identical in tint and intensity. A few results of estimations carried out with *N*-acetylchondrosamine solutions are given in Table II.

A comparison between the results obtained with the original method of Zuckerkandl and Messiner-Klebermass and those obtained using the new technique is shown in Table III. In each series of determinations the results of

Table III.

Experiment no.	Zuckerkandl and Messiner-Klebermass's method 2·00 mg. acetylglucosamine		New technique 0·500 mg. acetylglucosamine	
	Found mg.	% error	Found mg.	% error
1	2·12	+6·0	0·502	+0·4
2	2·16	+8·0	0·498	−0·4
3	1·95	−2·5	0·500	0·0
4	1·95	−2·5	0·498	−0·4
5	2·10	+5·0	0·492	−1·6
6	1·98	−1·0	0·502	+0·4
7	2·18	+9·0	0·500	0·0
8	1·94	−3·0	0·500	0·0
9	1·91	−4·5	0·490	−2·0
10	2·03	+1·5	0·502	+0·4
11	2·00	0·0	0·505	+1·0
12	2·08	+4·0	0·500	0·0

twelve consecutive estimations are given. It will be seen that with the old method the variation between the individual results is of the same order as that observed by Zuckerkandl and Messiner-Klebermass and may exceed 5 %. The results obtained with the modified technique show a variation of only 1–2 % from the true value.

Table IV. *The influence of foreign substances upon the accuracy of the estimation.*

Substance	Added mg.	*N*-Acetylglucosamine hydrochloride: Present mg.	*N*-Acetylglucosamine hydrochloride: Found mg.	% error
Glucose	1·0	0·500	0·500	0·0
,,	,,	,,	0·505	+1·0
,,	2·5	0·250	0·245	−2·5
Arabinose	1·00	0·500	0·500	0·0
,,	,,	,,	0·500	0·0
Fructose	1·00	0·500	0·500	0·0
,,	,,	,,	0·505	+1·0
,,	2·50	0·250	0·240	−4·0
Alanine	1·00	0·500	0·510	+2·0
,,	,,	,,	0·500	0·0
Histidine	,,	,,	0·495	−1·0
,,	,,	,,	0·510	+2·0
Glucosamine hydrochloride	,,	,,	0·505	+1·0
,,	,,	,,	0·500	0·0
1-Aminoglucose	,,	,,	0·500	0·0
,,	,,	,,	0·510	+2·0
N-Acetyl-1-aminoglucose	,,	,,	0·500	0·0
,,	,,	,,	0·505	+1·0

The influence of foreign substances.

Various sugars and amino-acids have been added to known amounts of *N*-acetylglucosamine in order to ascertain whether the presence of these substances has any influence upon the accuracy of the estimation. A few results of these tests are given in Table IV from which it will be seen that none of the substances added interfered with the determination.

Certain pyrrole and indole derivatives develop coloured solutions when treated with the *p*-dimethylaminobenzaldehyde reagent, and their presence in the solutions to be estimated will therefore influence the determination. Tryptophan does not appear to react with *p*-dimethylaminobenzaldehyde and does not yield a coloured solution under the conditions described, but the presence of moderate amounts (0·1–1·0 mg.) of this amino-acid in the *N*-acetylglucosamine solutions has been found to cause some delay in the rate of development of the reddish purple colour with resulting underestimation of the true result.

The influence of the time of heating and of the concentration of alkali on the subsequent colour development.

Estimations were made on solutions containing 0·5 mg. of *N*-acetylglucosamine per ml. The concentration of the alkali varied from 0·02 *N* to 2 *N* and three or four different periods of heating were tested with each alkali concentration. It is not necessary to give here the results obtained with all of these modifications but only to state that with concentrations of alkali higher than 0·5 *N* the solution, after heating at 100° for more than 2 minutes, developed a final colour intensity which indicated that a considerable destruction of either the *N*-acetylglucosamine or the corresponding oxazole derivative had taken place. The results obtained using 0·02 *N* and 0·1 *N* alkali are shown in Table V. It will be seen that the greatest colour intensity is attained when the *N*-acetylglucosamine

Table V. *Influence of the time of heating with various concentrations of alkali on the subsequent colour development.*

Alkali used	Concentration of alkali *N*	Time of heating min.	Colorimeter reading at maximum colour developed mm.
Na_2CO_3	0·02	5	9·0
,,	,,	10	**8·8**
,,	,,	15	8·9
,,	0·10	2	11·5
,,	,,	3	9·3
,,	,,	4	9·1
,,	,,	5	**9·0**
,,	,,	6	9·3
,,	,,	10	13·2
,,	,,	15	18·9
KOH	0·02	2	12·5
,,	,,	3	**10·1**
,,	,,	4	10·6
,,	,,	5	11·4
,,	0·10	2	10·2
,,	,,	3	13·2
,,	,,	4	20·6
Na_2HPO_4	0·10	5	21·6
,,	,,	10	17·2
,,	,,	30	14·8
,,	,,	40	**13·2**

is heated for 10 minutes at 100° in 0·02 *N* Na_2CO_3. If 0·02 *N* KOH is used in place of the 0·02 *N* Na_2CO_3 the maximum colour intensity which is reached for the same amount of *N*-acetylglucosamine is somewhat less. This is due to the action of the potassium hydroxide on the oxazole derivative, and the exact extent to which decomposition proceeds can readily be ascertained if a standard solution of the pure oxazole (IV) is treated with alkali under similar conditions.

The production of the oxazole derivative by heating with 0·02 *N* Na_2CO_3 appears to be almost quantitative. To show this a solution of maximum colour intensity which has been derived from a known amount of *N*-acetylglucosamine is compared colorimetrically with the colour developed by a solution of the pure oxazole which has been similarly treated with the *p*-dimethylaminobenzaldehyde reagent. Theoretically 0·5 mg. of *N*-acetylglucosamine should yield 0·459 mg. of the oxazole derivative. After treatment with 0·02 *N* Na_2CO_3 as described above, 0·5 mg. of *N*-acetylglucosamine gave a solution the colour intensity of which was equal to the colour given by 0·440 mg. of the oxazole; the conversion was therefore 96 % complete.

The production of a closed ring structure by the action of very low concentrations of alkali suggested that the reaction would probably take place at an alkalinity which could be considered to be within the range necessary for normal biological processes. The results obtained when *N*-acetylglucosamine was heated with 0·1 *N* Na_2HPO_4 (p_H 8·4) are shown in Table V. It will be seen that approximately 65 % of the *N*-acetylglucosamine has been converted into the oxazole derivative after 40 minutes' heating at 100°. A sterile solution of *N*-acetylglucosamine which has been buffered at p_H 8·4 with sodium phosphate and kept at 37° showed after 48 hours that about 10 % had been converted into the oxazole.

Although the maximum colour intensity reached per mg. of *N*-acetylglucosamine is slightly less when 0·1 *N* Na_2CO_3 is used in place of 0·02 *N*, we consider that for practical purposes a higher concentration of alkali than 0·02 *N* is more reliable since it is then possible to neglect slight traces of acid when these are present in the solution to be estimated.

The influence of the concentration of the hydrochloric acid upon the colour intensity.

A series of eight test-tubes containing *N*-acetylglucosamine (0·5 mg.) dissolved in 1·0 ml. of water was heated with 0·1 ml. of 0·5 *N* Na_2CO_3 for 5 minutes in a boiling water-bath and cooled. The tubes were then treated one at a time in the following manner. About 7·0 ml. of glacial acetic acid were added to each together with 0·1 ml. 0·5 *N* HCl and 1·0 ml. of *p*-dimethylaminobenzaldehyde (2 %) dissolved in glacial acetic acid which contained in the different experiments concentrations of hydrochloric acid[1] varying from 1 to 40 %. The tubes, after filling to the 10 ml. mark with acetic acid, were thoroughly shaken and the intensity of the colour produced was followed in each case by means of colorimetric observations at prearranged intervals against a stable artificial colour standard for a period of 90 minutes. The results are shown graphically in Fig. 1. It is seen that a reduction in the concentration of hydrochloric acid from 40 to 2·5 % in the *p*-dimethylaminobenzaldehyde reagent causes a steady increase in the intensity of the maximum colour produced; the rate of colour development is however slower with the lower acid concentrations and the colour formed is considerably more stable. When the reagent contains 20 or 30 % HCl, the

[1] Expressed throughout as % of concentrated HCl A. R. present.

intensity of the reddish purple colour reaches its maximum in less than 10 minutes and then rapidly fades. A concentration of hydrochloric acid of 5 % in the reagent causes the colour intensity to develop for about 45 minutes and then remain practically constant for a further 60 minutes. The colour development when 2·5 % of hydrochloric acid is employed is very slow, and the intensity of the colour developed does not reach its maximum until after about 90 minutes have elapsed; 1·0 % of acid in the reagent produces only a trace of colour after 2 hours. It should be emphasised however that the rate of colour development increases rapidly with increasing temperature and that the curves shown were obtained within the temperature range 13–16°.

From these observations it appears that the colour is very sensitive to an excess of hydrochloric acid and that, with concentrations of acid exceeding 0·5 % in the final acetic acid solution, fading of the colour goes on concurrently with colour production.

A similar series of curves is obtained when standard solutions of the pure oxazole derivative (IV) are treated in the same manner with the *p*-dimethylaminobenzaldehyde reagent containing different concentrations of hydrochloric acid.

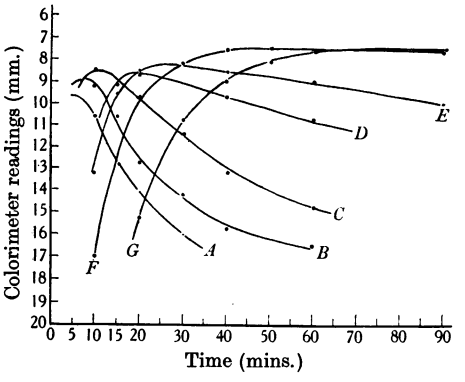

Fig. 1.

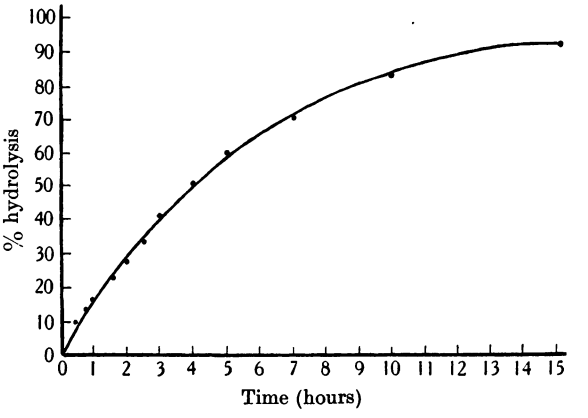

Fig. 2.

Fig. 1. The influence of the concentration of hydrochloric acid on the rate and intensity of colour development. Curve *A* 40 %, *B* 30 %, *C* 20 %, *D* 15 %, *E* 10 %, *F* 5 %, *G* 2·5 % of hydrochloric acid in the *p*-dimethylaminobenzaldehyde reagent.

Fig. 2. Curve showing rate of hydrolysis of *N*-acetylglucosamine with 0·1 *N* sulphuric acid.

Application.

The method has been used to follow the rate of hydrolysis of the acetyl group in *N*-acetylglucosamine, and the results of a typical hydrolysis experiment are shown in Fig. 2. The final figure shows that 94 % of the acetyl groups had been eliminated in 15 hours, and this figure was confirmed by estimation of the glucosamine formed using the colorimetric method described by Elson and Morgan [1933, 1]. Similarly the rate of esterification of *N*-acetylglucosamine to yield the non-reducing 1-methyl-*N*-acetylglucosamine and the hydrolysis of this compound back to *N*-acetylglucosamine can be readily followed by the colorimetric method described. The procedure has also been used to determine the rate at which the *N*-acetylhexosamine units, which are contained in the specific polysaccharide of *B. dysenteriae* (Shiga), are liberated by acid or enzymic hydrolysis [Morgan, 1931; 1932; Morgan and Thaysen, 1933].

SUMMARY.

A colorimetric method for the determination of *N*-acetylglucosamine, which was elaborated by Zuckerkandl and Messiner-Klebermass and which is based on a reaction originally described by Müller, has been studied. The procedure recommended by them has been found to give unsatisfactory results. By changing the technique it is possible to obtain reproducible results and at the same time to reduce considerably the amount of substance necessary for the determination. *N*-Acetylchondrosamine can be estimated by the modified method.

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